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Oxidant-induced formation of a neutral flavosemiquinone in the Na^+-translocating NADH:Quinone oxidoreductase (Na^+-NQR) from *Vibrio cholerae*

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ABSTRACT

The Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) from the human pathogen *Vibrio cholerae* is a respiratory flavo-FeS complex composed of the six subunits NqrA–F. The Na^+-NQR was produced as His_6-tagged protein by homologous expression in *V. cholerae*. The isolated complex contained near-stoichiometric amounts of non-covalently bound FAD (0.78 mol/mol Na^+-NQR) and riboflavin (0.70 mol/mol Na^+-NQR), catalyzed NADH-driven Na^+ transport (40 nmol $Na^+ min^{-1}$ mg^{-1}), and was inhibited by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide. EPR spectroscopy showed that Na^+-NQR as isolated contained very low amounts of a neutral flavosemiquinone (10^{-3} mol/mol Na^+-NQR). Reduction with NADH resulted in the formation of an anionic flavosemiquinone (0.10 mol/mol Na^+-NQR). Subsequent oxidation of the Na^+-NQR with ubiquinone-1 or O_2 led to the formation of a neutral flavosemiquinone (0.24 mol/mol Na^+-NQR). We propose that the Na^+-NQR is fully oxidized in its resting state, and discuss putative schemes of NADH-triggered redox transitions.

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1. Introduction

Flavins are versatile redox cofactors which catalyze the transfer of one or two electrons in many different enzymatic reactions [1]. In respiratory NADH dehydrogenases, the electrons from NADH are transferred via flavins and FeS centers to quinone. This exergonic redox reaction is coupled to the uphill transport of protons or Na^+ across the inner membrane of mitochondria or bacteria [2–5]. The pathogenic bacterium *Vibrio cholerae* contains a Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) which maintains an electrochemical Na^+ gradient across the inner membrane and influences the production of virulence factors [6,7]. The Na^+-NQR consists of six subunits, NqrA–F, and contains one [2Fe–2S] cluster, two covalently bound FMNs, one non-covalently bound FAD and ubiquinone-8 as prosthetic groups [3–7]. The presence of an additional riboflavin cofactor in the Na^+-NQR is discussed controversially [8,9]. The electron transfer pathway in the Na^+-NQR starts with a hydride transfer from the substrate NADH to the non-covalently bound FAD on the flavin domain of the NqrF subunit [10], followed by one-electron transfers to the vertebrate-type [2Fe–2S] cluster in the N-terminal domain of NqrF [11]. How electron transport from this FeS center to other flavin and ubiquinone cofactors in the complex and to the substrate quinone proceeds and how this reaction is coupled to Na^+ transport is still enigmatic.

For the highly related Na^+-NQRs from *V. cholerae* [12,13] and *Vibrio harveyi* [9] purified under oxic conditions, a resting state was proposed in which the enzymes contained near-stoichiometric amounts of a neutral (or "blue") flavosemiquinone ($FlH^{\cdot}$). This was intriguing since most flavoprotein radicals are rather stable under anaerobic conditions but rapidly react with molecular oxygen [14]. Moreover, the flavin radical species is usually not intrinsic to the system but must be generated by reduction [15]. It is therefore rather unlikely to obtain an enzyme containing a flavin radical from a purification performed in the presence of O_2. Notable exceptions are the neutral flavosemiquinones found in cryptochromes [16] and DNA photolyases [17]. The question whether the one-electron reduced state of the as isolated Na^+-NQR represents the resting state of the enzyme is important since schemes of redox events proposed for the closely related enzymes from *V. cholerae* [13] and *V. harveyi* [9] include the reduction of a pre-existing neutral flavosemiquinone to flavohydroquinone.

In the present study, comparative analysis of non-covalently bound flavins in the Na^+-NQR and its subcomplexes reveals that riboflavin is associated with the membranous part of the Na^+ pump. We show that anoxically purified Na^+-NQR contains only spurious amounts of organic radical(s). It is proposed that the redox cofactors of the Na^+-NQR are fully oxidized in the resting state of the Na^+ pump.

Abbreviations: Na^+-NQR, Na^+-translocating NADH:quinone oxidoreductase; DDM, *n*-dodecyl β-D-maltoside; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; FlH, neutral flavosemiquinone; $Fl^{\cdot-}$, anionic flavosemiquinone; Q-1, ubiquinone-1; SEC, size exclusion chromatography; TFA, trifluoroacetic acid; TCA, trichloroacetic acid

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2. Materials and methods

2.1. Cloning and expression of the Na^+-NQR

V. cholerae O395 N1 [18] served as a source of genomic DNA for cloning of the *nqr* operon. *Escherichia coli* DH5α served as a host for vector amplification. Genes *nqrA* to *nqrF* (bp 1998267–2004116 on chromosome I) were amplified from chromosomal DNA by PCR using the forward primer (CGA TAT ACA TAT GAT TAC AAT AAA AAA GGG ATT G) and the reverse primer (CGA TAG AAT TCT TAA CCA CCG AAG TCA TCC AG). PCR amplification by *Pfu*Turbo polymerase (Stratagene) was carried out as described by the manufacturer with an annealing temperature of 57 °C and an amplification time of 6 min. PCR products were digested with NdeI and EcoRI and ligated into the arabinose-inducible expression vector pEC422 [19], yielding vector pNQR1 encoding for Na^+-NQR comprising six histidine residues at the N-terminus of NqrA. Sequencing of pNQR1 confirmed the identity of the cloned *nqr* genes with the corresponding genomic sequence of *V. cholerae* O395 N1. Vector pNQR1 was introduced into *V. cholerae* O395 N1 lacking the *nqr* genes [7] by electroporation [20]. *V. cholerae* O395 N1Δ*nqr* transformed with pNQR1 was grown in medium containing 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 50 mM potassium phosphate (pH 7.3), 171 mM NaCl, 10 mM glucose, 50 μg ml^{-1} streptomycin and 200 μg ml^{-1} ampicillin. The inoculum was 20 ml of *V. cholerae* O395 N1Δ*nqr* transformed with pNQR1 added to 2 l prewarmed medium. The cells were grown aerobically in 5 l shaker flasks at 37 °C. Expression of Na^+-NQR was induced with 10 mM L-arabinose after an OD_{600} of 1 was reached. After induction, the growth temperature was decreased to 30 °C. Cells were grown overnight for 12 h, harvested and washed with 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl. Cell suspensions were frozen in liquid nitrogen and stored at −80 °C until use. Na^+-NQR used for reconstitution into proteoliposomes and Na^+ transport measurements was purified from cells which were washed with buffer containing K^+ instead of Na^+.

2.2. Purification of recombinant Na^+-NQR

Twenty gram wet cells were resuspended in 90 ml 50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl, 1.0 mM phenylmethylsulfonyl fluoride, 0.1 mM diisopropylfluorophosphate, 1.0 mM dithiothreitol, 5 mM $MgCl_2$, and traces of DNase I (Roche Diagnostics). If not indicated otherwise, subsequent steps were performed in an anaerobe chamber (95% N_2, 5% H_2 in the gas phase; Coy), or under a continuous stream of N_2. The cell suspension was passed twice through a French pressure cell at 7.58 MPa. Unbroken cells and large debris were removed by centrifugation at 35,000 ×*g* for 20 min. Soluble proteins were separated from the membrane fraction by ultracentrifugation (150,000 ×*g*, 1 h, 4 °C). The membranes were washed twice with buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 5% glycerol) and solubilized with 30 ml buffer A containing 5 mM imidazole and *n*-dodecyl β-D-maltoside (DDM; 0.5 g per g protein). After slight stirring for 30 min at 4 °C and ultracentrifugation (150,000 ×*g*, 1 h, 4 °C), the supernatant containing solubilized membrane proteins was loaded onto a Ni-nitrilotriacetic (NTA)-agarose column (5 ml bed volume, 1.4 cm diameter, Qiagen) equilibrated with buffer A containing 5 mM imidazole and 0.05% DDM. The column was washed with 25 ml buffer A containing 10 mM imidazole and 0.05% DDM. The Na^+-NQR was eluted with 25 ml buffer A containing 50 mM imidazole and 0.05% DDM and immediately dialyzed against buffer A. In some experiments, imidazole was removed by 20-fold dilution of Na^+-NQR with buffer B (50 mM sodium phosphate, pH 8.0, 5% glycerol) containing 0.03% DDM, followed by binding on a Fractogel TSK DEAE 650 column (3 ml bed volume, 1.4 cm diameter, Merck). The Na^+-NQR was eluted with 15 ml buffer A containing 0.05% DDM. To purify Na^+-NQR used in Na^+ uptake experiments, buffers contained K^+ instead of Na^+. Size exclusion chromatography (SEC) was performed with Na^+-NQR from the Ni-NTA chromatographic step which had been dialyzed and concentrated to approximately 10 mg ml^{-1} using Ultra-4 30K spin columns (cutoff, 30 kDa; Amicon). An aliquot of 100 μl was loaded onto a Superdex 200 10/300 GL column connected to an ÄKTA purification system (GE Healthcare). Elution was performed under air at 4 °C with 10 mM HEPES, pH 8.0, containing 5% glycerol, 300 mM NaCl and 0.05% DDM at a flow rate of 0.2 ml min^{-1}. The column was calibrated with molecular mass standards (Bio-Rad). The void volume of the column was determined with Blue Dextran 2000 (GE Healthcare).

2.3. Analytical methods

Protein was determined by the bicinchoninic acid method using the reagent from Pierce [21]. Bovine serum albumin served as the standard. SDS-PAGE was performed with 10% polyacrylamide [22] in the presence of 6 M urea [23]. The Na^+-NQR was allowed to react with sample buffer (50 mM Tris/HCl, pH 6.8, 5% SDS, 5.8% glycerol, 100 mM dithiothreitol and traces of bromphenol blue) for 30 min at room temperature to prevent precipitation of the hydrophobic NqrB, NqrD and NqrE subunits [23]. Thirty microgram protein was loaded per lane. Gels were inspected under UV illumination (excitation, 302 nm; emission, 520–620 nm; GeneGenius Bioimaging system, Syngene). Proteins were stained with Coomassie brilliant blue G-250.

2.4. Mass spectroscopy

Tryptic digestion was performed according to [24], modified as follows. Gel pieces from SDS-PAGE containing proteins stained with Coomassie were washed twice with 100 μl 100 mM NH_4HCO_3 in H_2O:acetonitrile (1:1) and once with 50 μl acetonitrile. Trypsin (100 ng) in 10 μl buffer (10 mM Tris, pH 8.2, 2 mM $CaCl_2$) was added to the gel pieces. 10 μl buffer was added and proteins were digested over night at 37 °C. The supernatant containing polypeptides was recovered and combined with extracts obtained by treatment of gel pieces twice with 100 μl 0.1% trifluoroacetic acid (TFA) in H_2O:acetonitrile (1:1). After evaporation of the solvent, the polypeptides were dissolved in 25 μl 0.1% formic acid. Aliquots of 0.5–2 μl were used for the analyses by electrospray tandem mass spectrometry (ESI/MS/MS) on a QTOF Ultima spectrometer connected to a nanoAcquity UPLC system (Waters). Matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI/MS/MS) was performed on a Ultraflex II mass spectrometer (Bruker). Aliquots of 10 μl were desalted using Ziptip C18 filters (Millipore) and mixed in a 1:1 ratio with matrix solution (5 mg ml^{-1} α-cyano-4-hydroxycinamic acid in 0.1% TFA and 50% acetonitrile). Protein database searches were performed using the Mascot program, version 2.2.0 (Matrix Science).

2.5. Extraction and analysis of non-covalently bound flavins

Protein solutions were concentrated to 1–4 mg ml^{-1} using Ultra-4 5K spin columns (cutoff 5 kDa, Amicon), and ice-cold trichloroacetic acid (TCA) was added to a final concentration of 6.25% [25]. After 5 min at 0 °C, precipitated proteins were removed by centrifugation (5 min, 15,800 ×*g*). The supernatant containing non-covalently bound flavins was neutralized by adding 0.8 M K_2HPO_4 and passed through PVDF filters (0.22 μm; Millipore). Flavins were separated on an EC 250/4 Nucleosil 120-5 C18 column (Macherey Nagel) connected to a HPLC system (Hewlett Packard). Elution was performed at 40 °C at a flow rate of 0.7 ml min^{-1} for 8 min with 1% (v/v) methanol in 0.05 M sodium acetate (pH 5.0), followed by a linear gradient from 1% to 50% (v/v) methanol in 0.05 M sodium acetate for 22 min and a washing step with 100% methanol (10 min). The absorbance at 450 nm was monitored. Standard solutions contained 120 pmol FAD, 37 pmol FMN and 56 pmol riboflavin (Sigma) and were quantified using the following extinction coefficients at 450 nm: FAD, 11.3 mM^{-1} cm^{-1} [25]; FMN, 12.5 mM^{-1} cm^{-1}, and riboflavin, 10.7 mM^{-1} cm^{-1} [26]. Flavin standards were added to flavins extracted from the Na^+-NQR, and the mixture was analyzed by HPLC (electronic supplementary material, Fig. S1). To exclude that extraction of non-covalently bound flavins of Na^+-NQR by TCA precipitation resulted in cleavage of FAD or FMN to riboflavin, flavin standards in buffer were treated with TCA in control reactions and analyzed by HPLC (electronic supplementary material, Fig. S1).

2.6. Enzyme tests

Na^+-NQR activities were determined in buffer (20 mM Tris/H_2SO_4, pH 7.5, 50 mM Na_2SO_4, 50 μg ml^{-1} bovine serum albumin) in the presence of 0.1 mM NADH and 0.1 mM ubiquinone-1 (Q-1; Sigma) using a diode array spectrophotometer (Hewlett Packard). The reaction was started by the addition of Na^+-NQR diluted in buffer. The absorbance at 340 nm, and the absorbance difference at 248 nm–268 nm, were followed simultaneously to monitor NADH oxidation and quinol formation, respectively [27].

2.7. Reconstitution of Na^+-NQR and Na^+transport

Na^+-NQR was reconstituted into proteoliposomes according to [27], modified as follows. Forty milligram L-α-phosphatidylcholine (from soybean, Type II-S, 14–23% as choline) were dissolved in 2 ml $CHCl_3$:CH_3OH (2:1) in a round bottom flask. The solvent was removed in a rotary evaporator, and the lipids were dried under vacuum for 4 h. Na^+-NQR from the Ni-NTA chromatographic step (1 mg in 0.3 ml) was added dropwise under gentle stirring until the lipid film was dissolved. Formation of proteoliposomes was achieved under air by slowly adding a 100-fold volume of buffer (10 mM Tris/HCl, pH 8.0, 100 mM K_2SO_4, 5% glycerol) under continuous stirring (30 drops min^{-1}). The proteoliposomes were collected by ultracentrifugation (150,000 ×*g*, 45 min) and resuspended in 3 ml buffer. Na^+ transport was determined under air at room temperature. Proteoliposomes (0.1 ml, 0.03 mg Na^+-NQR) were mixed with 0.2 ml buffer, and 2.5 mM NaCl was added. To inhibit Na^+ uptake, 0.1 mM 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) was allowed to react with the reconstituted Na^+-NQR for 20 min prior to NaCl addition. Transport was started by adding 0.1 mM Q-1 and 0.1 mM NADH at $t=0$. At indicated times, aliquots of 70 μl were applied to a 1 ml plastic syringe containing 0.3 ml Dowex 50 (K^+) and eluted with 0.8 ml of deionized water. The eluate was collected in plastic tubes, and the amount of Na^+ entrapped in the proteoliposomes was determined by atomic absorption spectroscopy.

2.8. Visible spectroscopy

The reduction and oxidation of anoxically prepared Na^+-NQR from the TSK DEAE 650 chromatographic step was followed in a cuvette sealed with a rubber stopper. NADH, sodium dithionite or Q-1 were added from stock solutions in the anaerobe chamber, and spectra were recorded on a Cary300Bio UV-Visible spectrophotometer (Varian) 10 min after each addition. To exchange buffers, the Na^+-NQR was passed through a NAP-10 column (GE healthcare) equilibrated with 50 mM sodium phosphate, pH 8.0, 5% glycerol, 300 mM NaCl, and 0.05% DDM in the anaerobe chamber. The concentration of the neutral flavosemiquinone was calculated from the absorption at 580 nm using an extinction coefficient $\varepsilon_{580}=4.5$ mM^{-1} cm^{-1} [28].

2.9. EPR spectroscopy

NADH (33 nmol) was added to 0.3 ml Na^+-NQR after TSK DEAE 650 chromatography (15.7 mg ml^{-1} in 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 5% glycerol, 0.34% DDM) in the anaerobe chamber. After 10 min, the sample was transferred to an EPR tube

and frozen in liquid N_2. An aliquot of Na^+-NQR without added NADH served as control. X-band EPR spectra were obtained with a Bruker ESP300 spectrometer with peripheral equipment and data handling as previously described [29]. The EPR spectra were simulated using the program EPR [30] assuming a spin=0.5 system. To determine the spin concentration in the Na^+-NQR, the total intensity of the simulated spectrum was compared with the intensity of the EPR spectrum of a $CuSO_4$ standard [29].

3. Results

3.1. Purification of Na^+-NQR

Fusion of a polyhistidine tag to the N-terminus of the peripheral NqrA subunit of the Na^+-NQR from *V. cholerae* allowed enrichment of the detergent-solubilized complex by Ni-NTA affinity chromatography. Subsequent size exclusion chromatography yielded a complex which eluted at an approximate molecular mass of 306 kDa and was termed NQR-1 (Fig. 1). We assumed that one copy of each Nqr subunit was present in the Na^+-NQR and calculated a theoretical molecular mass of 212 kDa for the complex. Taking into account the additional 50–76 kDa of a DDM micelle [31] which shields the hydrophobic part of the complex from the polar solvent, we conclude that NQR-1 mainly consists of monomeric Na^+-NQR. It is noteworthy that the *V. cholerae* Na^+-NQR with a polyhistidine tag at the C-terminus of the membrane-bound NqrF subunit exhibited an apparent molecular mass of 360 kDa according to SEC using the same detergent and under very similar conditions of salt and pH [7].

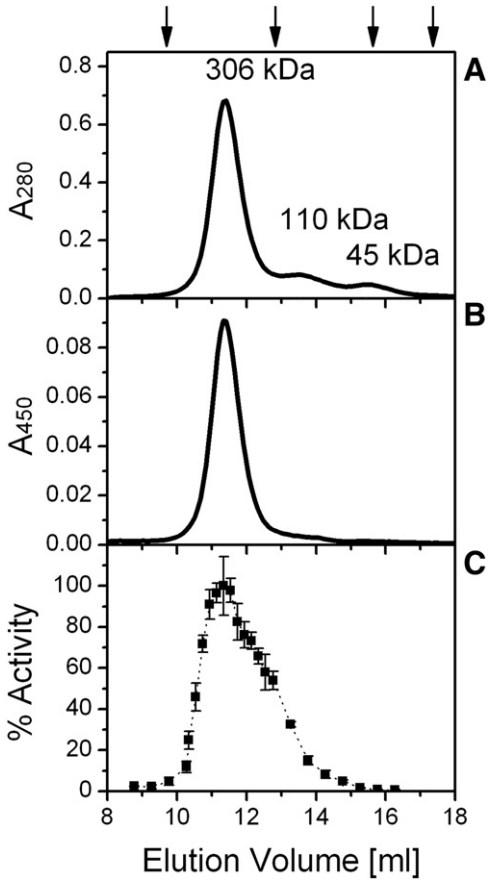

Fig. 1. Size exclusion chromatography of Na^+-NQR. Panel A, separation of 1 mg Na^+-NQR from the Ni-NTA chromatographic step monitored from the absorbance at 280 nm. Proteins eluting at 10.5–12.0 ml, 13.0–14.5 ml, and 15.0–16.5 ml with approximate masses of 306 kDa, 110 kDa and 45 kDa represented Na^+-NQR containing six subunits (NQR-1), a smaller subcomplex of the Na^+-NQR (NQR-2) and the isolated NqrA-His subunit, respectively (see Fig. 2). Arrows indicate the elution maxima of molecular mass standards (thyroglobulin, 670 kDa; γ-globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa). Panel B, flavin absorbance monitored at 450 nm. Panel C, NADH dehydrogenase activities of fractions were determined in triplicates. Hundred percent activity corresponds to 105 μmol min^{-1} mg^{-1}.

Table 1
Purification of the Na^+-NQR

Purification step	Protein [mg]	Specific activity [μmol mg^{-1} min^{-1}]	
		NADH dehydrogenase	Q-1 reductase
Membranes	1732	6.6±0.7	1.1±0.1
Solubilized membranes	1336	8.1±0.4	1.5±0.3
Ni-NTA chromatography	47	71.1±5.5	18.4±2.1
SEC NQR-1	24.5	104.9±19.1	21.4±9.4
SEC NQR-2	6.9	15.8±2.2	4.9±0.5
SEC NqrA-His	6.3	0.72±0.06	0.19±0.19

Starting material was 20 g *V. cholerae* cells. Size exclusion chromatography (SEC) yielded Na^+-NQR complex containing all six subunits (NQR-1), a smaller subcomplex of the Na^+-NQR (NQR-2), and the isolated NqrA-His subunit with apparent molecular masses of 306 kDa, 110 kDa and 45 kDa, respectively. Activities represent mean values from 3 measurements.

Upon purification, the specific NADH dehydrogenase and Q-1 reductase activities increased from 7 U mg^{-1} and 1 U mg^{-1} in membranes to 105 U mg^{-1} and 21 U mg^{-1} observed with purified NQR-1 (Table 1). NQR-1 contained all six Nqr subunits, as confirmed by mass spectroscopic analyses of tryptic digests of prominent polypeptides (Table 2) separated by SDS-PAGE (Fig. 2). Fluorescence emission of NqrB and NqrC under denaturing conditions (Fig. 2) demonstrated that both subunits contained covalently attached FMN, in accordance with previous reports [32,33]. The overall purification yield was 25 mg NQR-1 from 1.7 g membrane protein, or 20 g *V. cholerae* cells. We also obtained a smaller subcomplex of the Na^+-NQR (NQR-2), indicating partial dissociation of the complex before or during SEC. NQR-2 eluted from the SEC column at an apparent molecular mass of 110 kDa and exhibited approximately 15% of the specific NADH dehydrogenase activity of NQR-1 (Fig. 1). According to SDS-PAGE, NQR-2 contained NqrA-His, NqrF, NqrB and NqrC but lacked the highly hydrophobic NqrD and NqrE subunits (Fig. 2). NADH oxidation by NQR-2 most likely was catalyzed by NqrF. We previously showed that NqrF devoid of its single transmembrane helix oxidized NADH with Q-1 as artificial electron acceptor in the absence of other Nqr subunits [10]. Another protein fraction which eluted from the SEC column at an apparent mass of 45 kDa (Fig. 1) contained isolated NqrA-His, as confirmed by mass spectroscopy of tryptic digests and immunodetection of the polyhistidine tag after Western blot using an anti-His antibody (not shown). NqrA represents the only subunit of the Na^+-NQR complex which is not membrane-bound [34]. Major contaminants co-eluting with NqrA-His were the NAD-dependent malic enzyme (SwissProt

Table 2
MS/MS identification of subunits of the Na^+-NQR

SwissProt accession	Protein	Predicted mass (Da)	Number of peptide matches	
			MALDI MS/MS	ESI MS/MS
Q9KPS1	NqrA	48,624	29	n.d.
Q9KPS2	NqrB	45,357	4	1
Q9X4Q5	NqrC	27,619	2	6
Q9X4Q6	NqrD	22,837	1	1
Q9X4Q7	NqrE	21,470	0	1[a]
Q9X4Q8	NqrF	45,067	0	6
Q9KUF1	50S ribosomal protein L13	15,990	2	4
Q9KSR8	NAD-dependent malic enzyme	62,153	2	n.d.

Na^+-NQR from size exclusion chromatography eluting at 10.5–12.0 ml (NQR-1) was analyzed.
n.d., not determined.
[a] One fragment of subunit NqrE was detected in the tryptic digests of subunits NqrB, NqrC and NqrD.

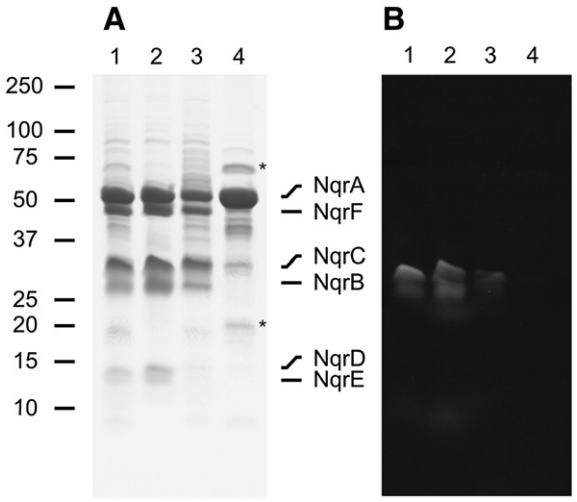

Fig. 2. SDS-PAGE of Na^+-NQR and detection of covalently bound flavins by fluorescence emission. Left, molecular mass standards (in kDa). Panel A, SDS-PAGE after staining with Coomassie; panel B, fluorescence emission of SDS-PAGE shown in panel A. Lane 1, Na^+-NQR after Ni-NTA chromatography. Na^+-NQR fractions from size exclusion chromatography are shown in lane 2 (10.5–12.0 ml, NQR-1), lane 3 (13.0–14.5 ml, NQR-2) and lane 4 (15.0–16.5 ml; NqrA-His). Nqr subunits were identified by MS/MS analyses of tryptic digests (Table 2). The NAD-dependent malic enzyme with an approximate mass of 60 kDa (SwissProt accession Q9KSR8) and the 50S ribosomal protein L13 with an approximate mass of 20 kDa (SwissProt accession Q9KUF1) are marked with asterisks (*).

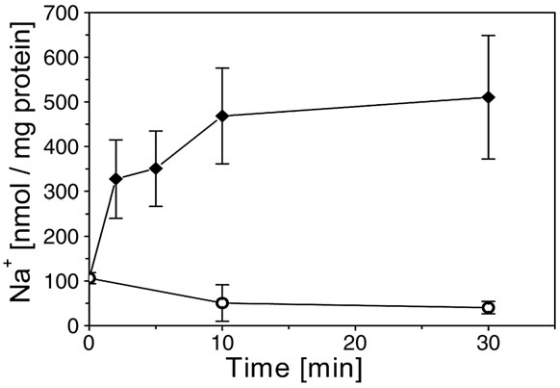

Fig. 3. NADH-driven Na^+ transport by Na^+-NQR. Na^+-NQR after Ni-NTA chromatography was reconstituted into proteoliposomes, and transport was started by adding 0.1 mM NADH and 0.1 mM Q-1. Na^+ entrapped in the proteoliposomes in the absence (closed symbols) or presence of 107 μM HQNO (open symbols) was determined by atomic absorption spectroscopy. Mean values from 3 experiments are presented.

accession Q9KSR8) and the 50S ribosomal protein L13 (SwissProt accession Q9KUF1) (Fig. 2). Both proteins do not contain flavin cofactors, and we did not detect extractable flavins (Table 3) or covalently bound flavins (Fig. 2) in NqrA-His in significant amounts. In accord with these findings, NqrA-His exhibited only very low NADH dehydrogenase activity (Table 1). Na^+-NQR from the Ni-NTA chromatographic step which was reconstituted into proteoliposomes catalyzed NADH-driven Na^+ transport at a rate of approximately 40 nmol Na^+ min^{-1} mg^{-1} (Fig. 3). Transport was abolished in the presence of HQNO, an inhibitor which is expected to interact with the ubiquinone binding site(s) of the Na^+-NQR. This demonstrates that Na^+-NQR with a polyhistidine tag attached to the N-terminus of the peripheral NqrA subunit functions as NADH-driven Na^+ pump.

3.2. Non-covalently bound flavins in Na^+-NQR

In 2002, Barquera et al. reported that riboflavin is an intrinsic component of the Na^+-NQR from *V. cholerae* and the highly conserved enzyme from *V. harveyi* [8]. For the *V. harveyi* Na^+-NQR, "the groups of Bogachev and Verkhovsky" confirmed the presence of non-covalently bound riboflavin associated with the complex but observed a ratio riboflavin:FAD of approximately 0.4–0.6 [9]. This is significantly lower than a ratio of 1 which is expected if both riboflavin and FAD are intrinsic cofactors of the Na^+-NQR. In addition, the authors found some non-covalently bound FMN (17% of the total FAD content) in the Na^+-NQR preparation and concluded that riboflavin and FMN associated with the Na^+-NQR from *V. harveyi* resulted from partial hydrolysis of the covalently bound FMN(s) during TCA precipitation [9].

We first checked the stability of FAD and FMN during incubation with TCA in control reactions following our protocol used for extraction of flavins from the Na^+-NQR. The HPLC chromatograms showed that no hydrolysis of FAD or FMN occurred under our experimental conditions (electronic supplementary material, Fig. S1). Next, the content of TCA-extractable (non-covalently bound) flavins in the Na^+-NQR was determined. A previously described analysis of extractable flavins in the *V. cholerae* Na^+-NQR was performed with enzyme after Ni-NTA affinity chromatography [8]. We performed an additional size exclusion chromatographic step which allowed comparing the content of extractable flavins in the Na^+-NQR from different purification stages (Fig. 4). The ratio riboflavin:FAD was close to 1, and the content of FAD (0.78 mol/mol Na^+-NQR) and riboflavin (0.70 mol/mol Na^+-NQR) in enzyme purified by SEC was higher than the flavin content of the enzyme after affinity chromatography (0.37 mol FAD/mol and 0.34 mol riboflavin/mol) (Table 3). These molar ratios are based on protein content, assuming that the Na^+-NQR has a molecular mass of 212 kDa. We did not detect extractable FMN in Na^+-NQR at any purification stage (Fig. 4). Both FAD (0.85 nmol mg^{-1}) and riboflavin (0.63 nmol mg^{-1}) were found in NQR-2, the smaller subcomplex of the Na^+-NQR consisting of NqrA-His, NqrF, NqrB and NqrC (Table 3). We confirmed that the isolated NqrF subunit devoid of its single transmembrane helix [10] exclusively contained non-covalently bound FAD which was not hydrolysed to FMN or riboflavin during TCA extraction (data not shown). Only spurious amounts of flavins were found in NqrA-His (Fig. 2, Table 3). These results indicated that the riboflavin detected in NQR-2 was bound to the NqrB or NqrC subunit. Note that these two subunits also contained covalently attached FMN (Fig. 2, [32,33]).

Table 3
Non-covalently bound flavins of Na^+-NQR and its subcomplexes

	FAD [nmol mg^{-1}] (mol/mol Na^+-NQR)	Riboflavin [nmol mg^{-1}] (mol/mol Na^+-NQR)	Ratio FAD: riboflavin
Ni-NTA	1.75 (0.37)	1.60 (0.34)	1.11
NQR-1	3.70 (0.78)	3.31 (0.70)	1.12
NQR-2	0.85	0.63	1.35
NqrA-His	0.04	0.03	1.33

Results from a representative purification are shown. Na^+-NQR after Ni-NTA chromatography was fractionated by size exclusion chromatography to yield Na^+-NQR containing all six Nqr subunits (NQR-1), a subcomplex containing NqrA-His, NqrF, NqrB, NqrC (NQR-2), and NqrA-His. Non-covalently bound flavins were extracted and quantified by HPLC as described in Materials and methods. Molar ratios (mol flavin/mol Na^+-NQR) are based on the calculated molecular mass of 212 kDa of a complex containing one copy of each Nqr subunit.

3.3. Flavin radicals in Na^+-NQR as isolated and after reduction with NADH

EPR spectroscopy allowed the characterization and quantification of organic radicals in anoxically purified Na^+-NQR before and after addition of NADH. In the absence of an electron donor, a signal

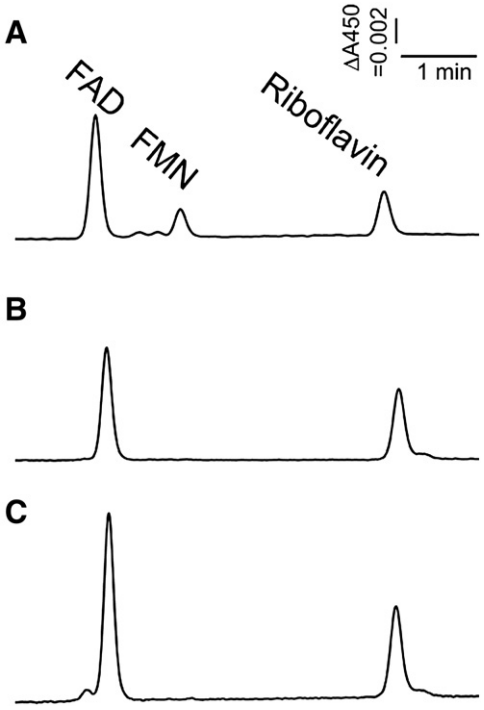

Fig. 4. Co-purification of riboflavin with the Na^+-NQR complex. Non-covalently bound flavins were extracted and analyzed by HPLC as described in Materials and methods. Relevant parts of the chromatograms (24–30 min) are shown. Trace A, flavin standards (120 pmol FAD, 37 pmol FMN, 56 pmol riboflavin). Trace B, non-covalently bound flavins from 60 μg Na^+-NQR after Ni-NTA chromatography. Trace C, non-covalently bound flavins from 134 μg Na^+-NQR (NQR-1) after size exclusion chromatography.

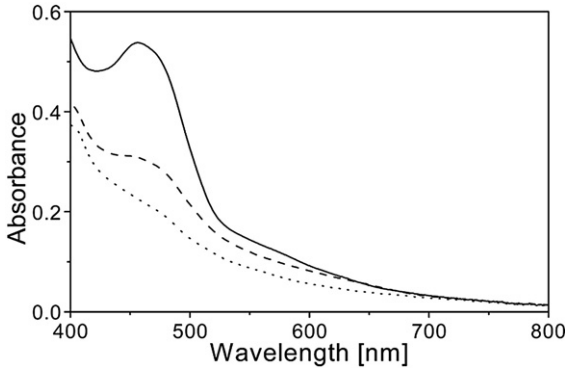

Fig. 6. Reduction of Na^+-NQR monitored by VIS spectroscopy. Solid trace, Na^+-NQR from Ni-NTA affinity chromatography (3.1 mg ml^{-1}) in 50 mM sodium phosphate, pH 8.0, 5% glycerol, 300 mM NaCl, and 0.05% DDM. Dashed trace, in the presence of 22 μM NADH; dotted trace, in the presence of 22 μM NADH and 1 mM sodium dithionite.

assigned to an organic radical was observed which was simulated using g_{xyz}=2.005 and a line width W_{xyz}=20 G (Fig. 5). Quantification of the simulated spectrum gave a spin concentration of 0.03 μM which was clearly substoichiometric compared to the concentration of Na^+-NQR (27 μM, based on FAD content). Upon reduction with NADH, there was a strong increase in intensity of the radical signal, and an additional line at g=1.94 appeared. The latter represents the g_{xy} region of the axial signal from the one-electron reduced [2Fe–2S] cluster located on subunit NqrF [10]. Quantification of a simulation of the EPR spectrum with g_{xyz}=2.004, W_{xyz}=15 G gave a radical concentration of 2.7 μM in NADH-reduced Na^+-NQR. In the Na^+-NQR from *V. cholerae*, the radical signal with a line width of 20 G observed in the as isolated enzyme without added NADH was assigned to a neutral flavosemiquinone, whereas the sharper signal with a line width of 15 G observed in the NADH-reduced Na^+-NQR was assigned to anionic flavosemiquinone(s) [13]. In this study performed with oxically purified Na^+-NQR, observed neutral or anionic flavosemiquinones were at near-stoichiometric levels with the enzyme [13]. In contrast, we found that the amount of neutral flavosemiquinone ($FlH^{\cdot}$) detected in Na^+-NQR purified under exclusion of O_2 was very low. Comparison of [$FlH^{\cdot}$] determined by EPR with the enzyme concentration based on FAD content gave a ratio of 10^{-3} mol $FlH^{\cdot}$/mol Na^+-NQR. In NADH-reduced Na^+-NQR, the organic radical assigned to the anionic flavosemiquinone ($Fl^{\cdot -}$) reached a ratio $Fl^{\cdot -}$:Na^+-NQR=0.1.

3.4. Oxidant-induced formation of a neutral flavosemiquinone in Na^+-NQR

Why did oxically prepared Na^+-NQR contain a neutral flavosemiquinone in stoichiometric amounts [13], whereas Na^+-NQR purified under exclusion of air did not? To address this question, we followed the reduction and reoxidation of the anoxically purified enzyme by visible spectroscopy. Addition of excess NADH to Na^+-NQR led to a decrease in VIS absorbance indicating partial reduction of flavin(s). Further reduction was achieved in the presence of dithionite. The

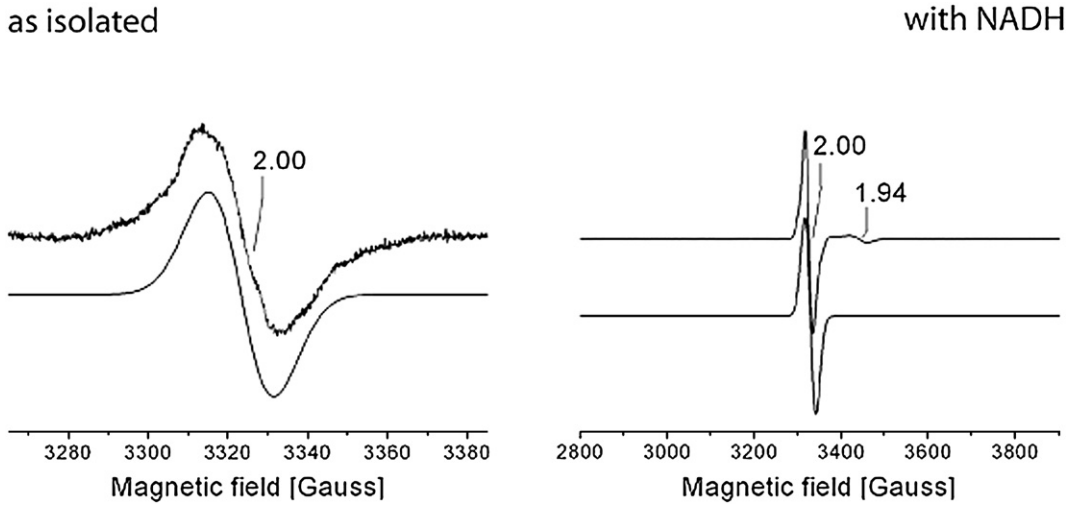

Fig. 5. Quantification of organic radicals detected in Na^+-NQR. EPR spectra (upper traces) of Na^+-NQR (15.7 mg ml^{-1}) as isolated (left panel) or in the presence of 110 μM NADH (right panel). Characteristic g-values are indicated. EPR conditions; temperature 70 K, microwave frequency 9.34 GHz, microwave power 2 mW. The lower traces are simulations of the EPR spectra with g_{xyz}=2.005, W_{xyz}=20 G (left panel) and g_{xyz}=2.004, W_{xyz}=15 G (right panel).

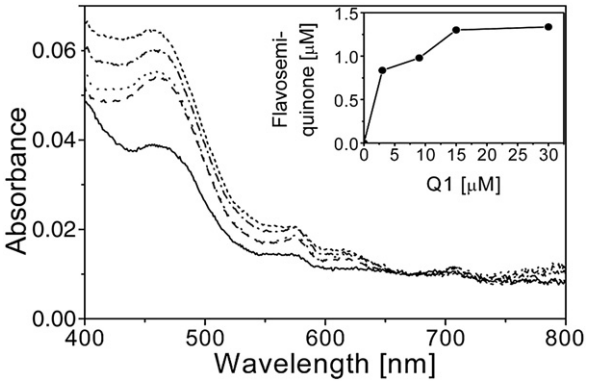

Fig. 7. Oxidation of Na^+-NQR with ubiquinone-1. Solid trace, Na^+-NQR (3.1 mg ml^{-1}) reduced with 150 μM NADH after buffer exchange by gel filtration. Reoxidation was achieved by adding Q-1 to concentrations of 3 μM, 9 μM, 15 μM and 30 μM. Absorbance differences at 580 nm between reduced and Q-1 oxidized Na^+-NQR were determined for each titration point to calculate the concentration of the neutral flavosemiquinone (inset).

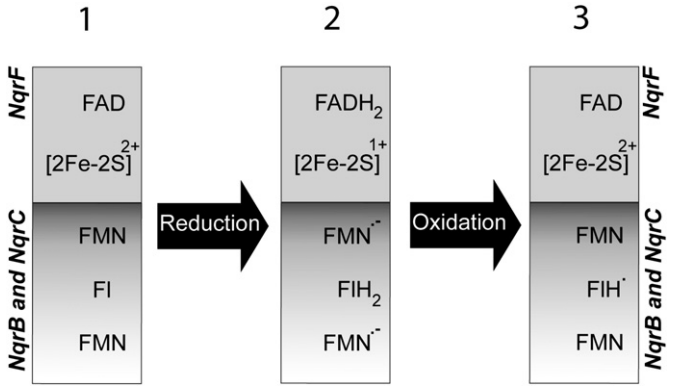

Fig. 9. Putative scheme of redox transitions of flavins in Na^+-NQR. (1) Subunits NqrF, NqrB and NqrC of the Na^+-NQR contain flavins in their oxidized state. NqrF is a peripheral subunit located at the cytoplasmic side of the Na^+-NQR complex. NqrB and NqrC are membrane-embedded subunits each containing one covalently attached FMN. An additional riboflavin (Fl) is bound to NqrB and/or NqrC. (2) The FAD on NqrF accepts a hydride from NADH and donates electrons to the [2Fe–2S] cluster located in close proximity. Intramolecular electron transfer to subunits NqrB and NqrC converts the FMNs to anionic flavosemiquinones ($FMN^{\cdot-}$), and the riboflavin to flavohydroquinone (FlH_2). (3) Oxidation of Na^+-NQR with ubiquinone-1 or O_2 yields the neutral flavosemiquinone $FlH^{\cdot}$.

reduced Na^+-NQR showed no absorbance in the 600 nm region expected for a neutral flavosemiquinone (Fig. 6). This is in accord with the EPR spectrum suggesting that $Fl^{\cdot-}$ rather than $FlH^{\cdot}$ is formed upon reduction of the Na^+-NQR by NADH (Fig. 5). Note that the anionic flavosemiquinone and the [2Fe–2S] center absorb in the same wavelength range [15], which obstructs the identification of $Fl^{\cdot-}$ in the Na^+-NQR by VIS spectroscopy.

We also monitored the oxidation of Na^+-NQR which had been reduced by NADH. Addition of Q-1 resulted in the formation of the neutral flavosemiquinone with characteristic maxima at 580 and 625 nm (Fig. 7). A maximum concentration of 1.3 μM $FlH^{\cdot}$ was observed in a solution containing 5.4 μM Na^+-NQR (based on FAD content) and 30 μM Q-1, corresponding to a ratio $FlH^{\cdot}$:Na^+-NQR = 0.24. Formation of the neutral flavosemiquinone was accompanied with an increase in absorbance around 800 nm which was reminiscent of a band previously observed in the Na^+-NQR from *V. alginolyticus* [35]. In this study, we tentatively assigned the 800 nm absorbance as due to the charge-transfer complex between reduced flavin and NAD^+ [36]. If O_2 instead of Q-1 was used as electron acceptor for the oxidation of the Na^+-NQR, $FlH^{\cdot}$ was also formed, but the absorbance at 800 nm was much weaker (Fig. 8). The EPR spectrum of NADH-reduced Na^+-NQR which was reoxidized by O_2 showed an organic radical with a line width of 20 G assigned to the neutral flavosemiquinone (data not shown). Quantification of the simulated EPR spectrum yielded a ratio $FlH^{\cdot}$:Na^+-NQR = 0.6 which is comparable to the amount of $FlH^{\cdot}$ detected by VIS spectroscopy. It is concluded that the isolated Na^+-NQR contained only traces of a neutral flavosemiquinone, but this one-electron reduced flavin accumulated when the partially reduced enzyme reacted with Q-1 or O_2.

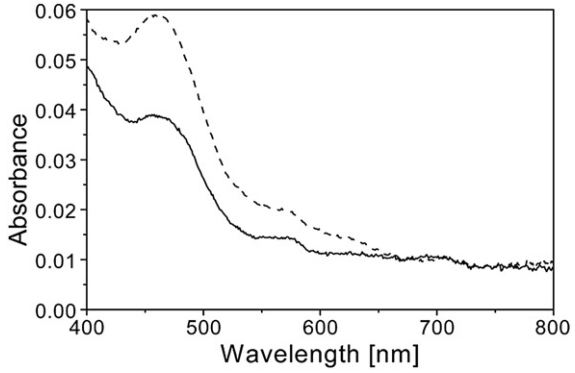

Fig. 8. Oxidation of Na^+-NQR with O_2. For experimental conditions, see legend to Fig. 7. Solid trace, Na^+-NQR after reduction with NADH and buffer exchange; dashed trace, after exposure to air for 90 min.

4. Discussion

The Na^+-NQR is a respiratory complex which contains one [2Fe–2S] cluster, one FAD, two covalently bound FMNs, and ubiquinone-8. The presence of an additional riboflavin in the highly related enzymes from *V. cholerae* and *V. harveyi* was discussed controversially [8, 9]. We produced recombinant Na^+-NQR from *V. cholerae* with a polyhistidine tag attached to its peripheral NqrA subunit and found 0.78 mol riboflavin per mol Na^+-NQR purified by size exclusion chromatography. Flavin analysis of a subcomplex of the Na^+-NQR consisting of the subunits NqrA-His, NqrF, NqrB and NqrC indicated that the riboflavin in the Na^+-NQR was bound to NqrB or NqrC.

To elucidate the mechanism of redox-driven Na^+ transport by the Na^+-NQR, the pathway of electrons through the complex must be described, and catalytically relevant redox transitions of the cofactors have to be identified. An important question concerns the redox state of cofactors in the Na^+-NQR prior to the reaction with NADH. Na^+-NQR from *V. harveyi* [9] and *V. cholerae* [7] purified in the presence of O_2 contained a neutral (or "blue") flavosemiquinone ($FlH^{\cdot}$). Our study shows that active Na^+-NQR from *V. cholerae* which was isolated under exclusion of O_2 contained only minor traces of flavin radicals. This indicates a resting state of the enzyme where the flavins and the FeS cluster are fully oxidized. Further studies will be required to investigate if the fully oxidized Na^+-NQR represents an intermediate in the catalytic cycle.

The peripheral NqrF subunit of the Na^+-NQR contains one FAD and one [2Fe–2S] cluster and represents the electron input module of the complex. Reduction of the isolated NqrF subunit with NADH yielded $FADH_2$ and the one-electron reduced FeS center [10]. Upon reduction of the Na^+-NQR complex with NADH, fully reduced flavin(s), anionic flavosemiquinone(s), and the reduced [2Fe–2S] center were observed. Barquera et al. showed that the anionic flavosemiquinone observed in NADH-reduced Na^+-NQR [9,12] resulted from two distinct flavin species assigned to the FMNs on the membrane-bound NqrB and NqrC subunits [13]. Flavin cofactors on NqrB or NqrC are likely to accept electrons from the [2Fe–2S] center on subunit NqrF. A neutral flavosemiquinone accumulated when the NADH-reduced Na^+-NQR was reoxidized, suggesting that formation of $FlH^{\cdot}$ resulted from an $FlH_2 \rightarrow FlH^{\cdot}$ redox

transition (Fig. 9). Barquera et al. [13] tentatively assigned the neutral flavosemiquinone to the riboflavin cofactor located on subunit NqrB and/or NqrC. The location of the enzyme-bound ubiquinone-8, or the binding site for substrate quinone, is unknown, but there is evidence that NqrB is part of the quinone reductase module of the complex [37].

Our study indicates that four flavins in the Na^+-NQR are converted to the partially or fully reduced state when the enzyme reacts with NADH (Fig. 9). Two non-covalently bound flavins (FAD and riboflavin) preferentially act as two-electron mediators, whereas two covalently bound FMNs undergo one-electron transitions ($FMN/FMN^{\cdot -}$). In contrast, Bogachev and Verkhovsky proposed that upon reduction of the Na^+-NQR by NADH, three redox-active flavins undergo one two-electron transition ($FAD/FADH_2$) and two one-electron transitions ($Fl/Fl^{\cdot -}$ and $FlH^{\cdot}/FLH_2$, respectively) [4]. The authors proposed that the flavin which gives rise to the neutral flavosemiquinone is not stabilized in its oxidized state [4]. Our results do not support this notion, since formation of $FlH^{\cdot}$ required the redox transitions $Fl \rightarrow FlH_2 \rightarrow FlH^{\cdot}$ (Fig. 9). Future studies will address how the two covalently linked FMNs and the riboflavin on subunits NqrB and NqrC mediate electron transfer from the reduced [2Fe–2S] center on subunit NqrF to ubiquinone-8, and whether the formation or decay of flavin radical(s) in the Na^+-NQR is coupled to a Na^+ translocation step in the catalytic cycle. The characterization of the redox states of flavin cofactors in Na^+-NQR in the absence or presence of NADH, ubiquinone-8, and Na^+, will be the prerequisite to answer these questions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2008.04.006.

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